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Immunoglobulin M Antibody Test To Detect Genogroup II Norwalk-Like Virus Infection

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Sera obtained from adult volunteers inoculated with genogroup II Norwalk-like viruses (NLVs), Hawaii virus, and Snow Mountain virus and from patients involved in outbreaks of gastroenteritis were tested for genogroup II NLV Mexico virus-specific immunoglobulin M (IgM) by use of a monoclonal antibody, recombinant Mexico virus antigen (rMXV)-based IgM capture enzyme-linked immunosorbent assay (ELISA). Sera from genogroup I Norwalk virus (NV)-inoculated volunteers and from patients involved in a genogroup I NLV outbreak were also tested. In sera from those infected with genogroup I NV or NLVs in volunteer and outbreak studies, only 3 of 25 were rMXV IgM positive; in contrast, 24 of 25 were IgM positive for recombinant NV (rNV). In sera from those infected with genogroup II NLVs in volunteer and outbreak studies, 28 of 47 were rMXV IgM positive and none were IgM positive for rNV, showing the specificity of each IgM test for its respective genogroup. In an outbreak of gastroenteritis not characterized as being of viral etiology but suspected to be due to NV, 7 of 13 persons had IgM responses to rMXV, whereas none had IgM responses to rNV, thus establishing the diagnosis as genogroup II NLV infection. The rMXV-based IgM capture ELISA developed is specific for the diagnosis of genogroup II NLV infections.

In a recent classification, the family *Caliciviridae* comprises four genera: *Vesivirus*, *Lagovirus*, Norwalk-like viruses (NLVs), and Sapporo-like viruses (14). In previous reports, viruses in the NLV genus have been categorized in two genogroups. Genogroup I includes the prototype Norwalk virus (NV) and related viruses, and genogroup II includes viruses such as Snow Mountain virus (SMV), Hawaii virus (HV), and Mexico virus (MXV) (23, 24, 26, 31, 36). NV has been the most extensively studied, although it currently does not seem responsible for most gastroenteritis caused by NLVs (4, 23, 26, 34, 37). The development of recombinant NV (rNV) has provided a highly purified antigen for detecting immunoglobulin G (IgG) antibodies against NV and genogroup I NLVs (11, 12, 15, 21, 22, 32, 33). More recently, recombinant capsid antigen has been developed from MXV, a genogroup II NLV (24, 25). This recombinant MXV (rMXV) antigen has been used in several studies to detect IgG antibodies against genogroup II NLVs (7, 17, 25, 33, 34).

Testing for seroconversion with either of these recombinant antigens requires an early acute-phase serum sample and a convalescent-phase serum sample to detect a minimum four-fold rise in IgG antibody levels required to diagnose infection. We recently described a monoclonal antibody, recombinant antigen-based IgM capture enzyme-linked immunosorbent assay (ELISA) for the detection of specific IgM antibodies to NV (1). We found that IgM antibodies to NV developed by 8 days after exposure and were not detectable in normal sera even if high titers of IgG antibodies were present. NV-specific IgM antibodies were not detected in sera from SMV- or HV-inoculated volunteers (1). Two studies have used immune electron microscopy to detect IgM to genogroup II NLVs (3, 30), and IgM responses to rMXV antigen in sera from persons infected with small round structured viruses have been reported (17). The purpose of this study was to determine the efficacy of a monoclonal antibody, rMXV-based IgM capture ELISA for the diagnosis of genogroup II NLV infections.

MATERIALS AND METHODS

Serum samples. Sera were obtained from patients in volunteer studies and in outbreaks of gastroenteritis. For sera from individuals known to be infected with genogroup I NLVs, nine paired sera were from NV-inoculated volunteers who had been infected with the 8FIIa strain and were shown to be positive by seroconversion and by an IgM ELISA for NV (1). Sixteen paired sera from an outbreak of gastroenteritis in Erie County, New York, and originally diagnosed as NV positive by human reagent-based antigen and antibody assays were tested (10). By genotyping (1), the outbreak from which the sera used here were obtained was shown to be associated with a genogroup I NLV (V Ward 1/90).

Sera from patients infected with genogroup II NLVs included paired sera obtained in two HV-inoculated volunteer studies with two volunteers each (unpublished data), along with three convalescent-phase sera from SMV-inoculated volunteers (obtained from R. Dolin, University of Rochester). Both groups consisted of individuals who became ill. Sera from outbreaks included 21 paired sera from an outbreak of SMV in a New York City high school cafeteria in 1985 (16). This outbreak involved approximately 600 students and cafeteria workers. Acute-phase sera were collected 4 to 8 days after the onset of symptoms, and convalescent-phase sera were collected 2 weeks later. Two paired sera from individuals infected with Taunton virus (TNV) (2) were obtained from D. Lewis, Leeds, United Kingdom. Sera collected during investigations of two additional outbreaks were also tested. Five paired sera and eight single convalescent-phase sera were obtained from patients involved in an outbreak at a nursing home (University of Massachusetts Medical Center—University Commons [UMMC-UC]) in 1996 (unpublished data). During a 2-week period, 68 residents and staff members became ill with gastroenteritis. Routine examination for bacterial and parasitic agents by the University of Massachusetts Medical Center clinical microbiology laboratory yielded negative results. Stool samples from three patients were tested by reverse transcription-PCR for NLVs. The portion sequenced (57 bases) had 95% identity in the polymerase region with MD-V6, a genogroup II NLV involved in an outbreak in a Maryland nursing home in 1987 (28) (accession no. MCU07613), and 98% identity with Halifax NLV (unpublished data) (accession no. NLU87651). The second outbreak involved patients who developed gastroenteritis after a Rhode Island graduation banquet in 1986

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(unpublished data). Forty-one of 93 persons at the banquet became ill. No NV was detected by an ELISA for NV antigen (20) in 12 stool samples from persons involved in the outbreak. Acute-phase sera were collected 6 days after the banquet, and convalescent-phase sera were collected 7 weeks later. Thirteen paired sera were available; 3 of 13 seroconverted to NV in a blocking radioimmunoassay for NV. These outbreaks were tested with both MXV and NV IgG and IgM assays to determine the utility of these tests in outbreaks that were not characterized with regard to viral etiology.

Paired sera were obtained from four adults involved in a documented MXV outbreak. The outbreak was confirmed as MXV by reverse transcription-PCR and sequencing of virus in stool samples (unpublished data). These sera also served as positive controls for the rMXV-based IgM test.

Six paired sera were obtained from adults with astrovirus gastroenteritis (19), and four paired sera were obtained from adults with rotavirus gastroenteritis (8). These were tested with both MXV and NV IgG and IgM assays as controls for the specificity of the assays for sera from persons with viral gastroenteritis due to viruses other than NLVs.

Eighty normal human sera were obtained from a group of adult donors from the University of Massachusetts Medical Center hospital blood bank and from children admitted to the hospital for reasons other than gastroenteritis. All of the above sera were stored at ≤ -20°C.

Monoclonal antibody to rMXV. A mouse monoclonal antibody to rMXV was developed by procedures previously described (18, 27). Briefly, two inoculations of rMXV in Titer-Max adjuvant (CytRx Corporation, Norcross, Ga.) were given subcutaneously to BALB/c mice. After sufficient titers were obtained (1:32,000 in an ELISA against rMXV), the spleen cells were fused to SP2/0-Ag14 mouse myeloma cells. An ELISA was used to screen for hybridoma cells producing anti-rMXV antibodies. The antibodies were confirmed as anti-rMXV antibodies by a blocking antibody test with human convalescent-phase serum from a patient who had an MXV infection. The hybridoma selected was designated clone 1B5 and was isotype IgG2a.

IgM capture antibody ELISA. Polyvinyl microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with unlabeled rabbit anti-human IgM (Fc5μ) (Accurate Chemical, Westbury, N.Y.) at 0.25 μg/50 μl of 0.1 M phosphate-buffered saline (PBS) per well. The plates were incubated at 37°C for 2 h, washed three times (with PBS plus 0.15% Tween 20), and blocked overnight at 20 to 22°C with 5.0% bovine serum albumin and 0.25% Bloom 60 gelatin (Sigma Chemical Co., St. Louis, Mo.) in PBS. The plates were washed three times, and duplicate twofold serial dilutions of human serum starting at a 1:25 dilution were made with 50% fetal bovine serum (FBS)–50% 0.025 M Tris-HCl buffer (pH 7.2) (FBS–Tris-HCl buffer) containing 0.015% Tween 20 (50 μl per well) and incubated for 1 h at 37°C. The plates were washed five times, and 50 ng of rMXV in 50 μl of FBS–Tris-HCl buffer was added to each well of one of the duplicate rows. To the second row, 50 μl of FBS–Tris-HCl buffer without rMXV was added. After overnight incubation at 20 to 22°C, the plates were washed five times, and 50 μl of a 1:5,000 dilution of monoclonal antibody to rMXV in FBS–Tris-HCl buffer was added per well and incubated for 1 h at 37°C. The plates were washed, and peroxidase-labeled goat anti-mouse IgG (heavy and light chains) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) at 1 μg/ml in FBS–Tris-HCl buffer plus 1% normal rabbit serum was added and incubated for 1 h at 37°C. The plates were washed five times. The substrate for peroxidase (0.05 ml of *O*-phenylenediamine–H_2O_2; Abbott Laboratories, North Chicago, Ill.) was added and allowed to stand for up to 10 min, and the reaction was stopped with 0.1 ml of 1 N H_2SO_4. The A_{492} of the solution was measured with a plate reader spectrophotometer (Whittaker Bioproducts, Walkersville, Md.). A positive test was one in which a 1:25 dilution (the lowest tested) or higher gave an A_{492} of $\geq$0.200 above the A_{492} of the control (wells with no antigen added) and was $\geq$3 times the standard deviation of the mean A_{492} obtained with 10 prechallenge sera tested at a 1:25 dilution in wells with no antigen added. Detection of IgM to rNV was done as described previously (1).

IgG antibody ELISA. Alternate rows in polyvinyl microtiter plates were coated with rMXV at 50 ng/50 μl of 0.1 M PBS per well. The plates were incubated at 37°C for 4 h and washed three times, and all wells were blocked overnight as in the IgM capture antibody test. The plates were washed three times, and twofold serial dilutions of human serum starting at a 1:800 dilution were made with FBS–Tris-HCl buffer (50 μl per well) and incubated for 1 h at 37°C. Sera with titers of $<$1:800 were retested starting at a 1:100 dilution. Dilutions for each serum were made in both the rMXV-coated row and the control row. The plates were washed four times, and peroxidase-labeled goat anti-human IgG (heavy and light chains) (Kirkegaard & Perry Laboratories) at 1 μg/ml in FBS–Tris-HCl buffer was added and incubated for 1 h at 37°C. The substrate for peroxidase was added as described for the IgM capture antibody test, and the A_{492} of the solution was measured with a plate reader spectrophotometer. A positive test was one in which a 1:100 dilution (the lowest tested) or higher gave an A_{492} of $\geq$0.200 above the A_{492} of the control (wells with no antigen added) and was $\geq$3 times the standard deviation of the mean A_{492} obtained with 10 prechallenge sera tested at a 1:100 dilution in wells with no antigen added. Detection of IgG to rNV was done as described previously (1).

RESULTS

The results of the serological tests for MXV from volunteer studies and outbreaks are shown in Table 1. Among sera from those infected with genogroup I NLVs (combined volunteer and outbreak studies), only 3 of 25 were rMXV IgM positive; in contrast, 24 of 25 were IgM positive for rNV. In sera from those infected with genogroup II NLVs (combined volunteer and outbreak studies), 28 of 47 were rMXV IgM positive and none were IgM positive for rNV. These results show the specificity of the rMXV IgM test and confirm the specificity of the rNV IgM test previously reported (1). In an outbreak of gastroenteritis not characterized with regard to viral etiology but suspected to be due to NV (the Rhode Island graduation banquet), 7 of 13 persons had IgM responses to rMXV, whereas none had IgM responses to rNV. These results established the diagnosis as genogroup II NLV. All four sera from patients in a documented MXV outbreak were positive in the rMXV IgM test and negative in the rNV IgM test.

Among four paired sera from children with astrovirus infections and among six paired sera from adults with rotavirus infections, none showed seroconversion or were IgM positive for either rMXV or rNV (Table 1). Eighty sera from noninfected individuals ranging in age from 1 to 59 years were tested for IgG and IgM to rMXV (Table 2). The proportion of sera positive for rMXV IgG ranged from 45 to 90% for the different age groups. Two sera from children between 1 and 4 years of age were IgM positive for rMXV.

DISCUSSION

Several studies have shown the development of NV-specific IgM antibodies as a result of NV infection (1, 6, 9, 13, 29, 35), but reports on the development of IgM antibodies to genogroup II NLV infections are limited. Two earlier studies (3, 30) with immune electron microscopy showed IgM reactivity to viruses that were later shown to be genogroup II NLVs, and a recent report with an ELISA showed IgM responses to genogroup II NLVs involved in outbreaks (17). Sera from genogroup I NLV infections were not tested in that study. Our results with volunteer and outbreak sera show that the rMXV-based IgM capture ELISA that we developed detects antibodies to genogroup II viruses such as HV, SMV, TNV, and related viruses. IgM antibodies to rMXV were not detected in sera from NV-inoculated volunteers. Neither the rMXV IgM test nor the rNV IgM test reacted with sera from patients with rotavirus or astrovirus infections, further demonstrating the specificity of these tests for NLV infections.

During NV infections, IgM to NV has been found to be more specific for NV than IgG to NV (1, 28, 34). It is well established that repeated stimulation by a given antigen usually increases IgG titers but decreases specificity. This situation should not occur with the IgM response, because IgM is not associated with anamnestic responses to repeated antigen exposure. Thus, exposure to several related NLVs could result in IgG with a broader specificity, as was seen in the seroconversion data from the Erie County outbreak involving a genogroup I NLV (V Ward 1/90). It has been shown in a previous study that there is a correlation between seroconversion and NLV genotype, but it was suggested in that report that the IgM response could be more specific (33).

The MXV IgM test was most useful when used in combination with the NV IgM test for the outbreaks at UMMC-UC, the Rhode Island graduation banquet, and in Erie County. The lack of fourfold or greater rises in IgG antibody titers to rMXV from patients in the outbreak at UMMC-UC was probably due

TABLE 1. Detection by ELISA of IgG seroconversion (sc) and IgM antibodies specific for rMXV and rNV in volunteers and in natural outbreaks of gastroenteritis

Genogroup	Samples	No. of sera with the following result/no. tested:											
		IgM+[a] for:		IgG sc+ for:		IgM+ and IgGsc+ for:		IgM+ and IgGsc− for:		IgGsc+ and IgM− for:		IgGsc− and IgM− for:	
		rMXV	rNV	rMXV	rNV	rMXV	rNV	rMXV	rNV	rMXV	rNV	rMXV	rNV
I	NV-inoculated volunteers (paired sera)	0/9	9/9	1/9	9/9	0/9	9/9	0/9	0/9	1/9	0/9	8/9	0/9
	Erie County outbreak (paired sera)	3/16	15/16	10/16	13/16	0/16	12/16	2/16	3/16	9/16	1/16	2/16	0/16
II	HV-inoculated volunteers (paired sera)	2/4	0/4	4/4	1/4	2/4	0/4	0/4	0/4	2/4	3/4	0/4	1/4
	SMV-inoculated volunteers (convalescent-phase sera)	3/3	0/3	NA[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA
	SMV outbreak (paired sera)	11/21	0/21	4/21	1/21	2/21	0/21	9/21	0/21	2/21	1/21	8/21	20/21
	TNV outbreak (paired sera)	2/2	0/2	2/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2
	MXV outbreak (paired sera)[b]	4/4	0/4	4/4	1/4	4/4	0/4	0/4	0/4	0/4	1/4	0/4	3/4
	UMMC-UC outbreak (paired sera)[c]	2/5	0/5	0/5	1/5	0/5	0/5	2/5	0/5	0/5	1/5	3/5	4/5
	UMMC-UC outbreak (convalescent-phase sera)	4/8	0/8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Unknown	Rhode Island outbreak (paired sera)	7/13	0/13	2/13	1/13	1/13	0/13	6/13	0/13	1/13	1/13	5/13	12/13
Other[d]	Rotavirus (paired sera)	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6/6	6/6
	Astrovirus (paired sera)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4

[a] A serum positive for IgM is one in which the IgM antibody titer is ≥1:25 in a single sample.
[b] The outbreak was confirmed to be due to MXV.
[c] Acute-phase sera were collected late.
[d] All sera from patients with rotavirus and astrovirus infections had IgG titers to rNV and rMXV in their acute- and convalescent-phase sera.
[e] NA, not applicable.

to the late collection of the acute-phase sera. In paired sera from two patients at the Rhode Island graduation banquet, there were fourfold increases in IgG antibody titers to rMXV in both patients and to rNV in one patient. Both outbreaks were diagnosed by use of a combination of rNV- and rMXV-based IgM capture assays. The larger sample size of the SMV outbreak associated with eating in a school cafeteria also demonstrates the utility of the rMXV-based IgM test compared with seroconversion. Of the 13 persons found positive by either seroconversion or IgM, only 2 were missed by the rMXV-based IgM test, whereas 9 were missed by seroconversion.

Among 80 normal sera tested, sera from two children between 1 and 4 years old were rMXV-specific IgM positive. Occasional positive results may occur in the rMXV-based IgM test because high levels of IgG antibodies to MXV have been detected in young children (5, 7, 24, 34) and the IgM-positive sera could represent a recent infection. We did not have sufficient information available on volunteer or outbreak sera to determine the time between exposure to NLVs and the appearance of specific IgM. In a previous study on the detection of IgM antibodies to genogroup I NLV infections (1), rNV-specific IgM was not detected in volunteers by 5 days but was detected by 8 days (sera from days 6 and 7 were not obtained). In outbreak sera, rNV-specific IgM was detected 6 to 7 days after the estimated time of exposure (1), and it is likely that similar times for the appearance of virus-specific IgM would be required for other NLVs.

rNV- and rMXV-based IgM capture assays can be used to determine whether an outbreak is due to NLVs and are useful for genogroup classification in epidemiological studies. IgM assays are especially useful if acute-phase sera are collected late or if paired sera are not available.

TABLE 2. Prevalence of IgG and IgM to rMXV in 20 normal sera, as determined by an ELISA

Age range (yr)	No. of sera positive for:	
	IgG[a]	IgM[b]
1–4	12	2
5–17	9	0
18–29	18	0
30–59	18	0

[a] Titer of ≥1:100.
[b] Titer of ≥1:25.

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